REMARKS

Claims 1-50 are all the claims pending in the application. The Office withdraws claims 1-36, 41, 42, 45-47 and 49 as being drawn to a nonelected invention and/or species. Applicants amend claims 37, 38 and 48, support for which can be found at least in the originally filed claims and paragraphs 13 and 27 of the published specification. No new matter is added. Entry of the Amendment is kindly requested.

I. Formal Matters

The Applicants thank the Examiner for rejoinder of claim 40; for acknowledging Applicants' claim to priority under 35 U.S.C. 119(a)-(d); and for acknowledging Applicants' PTO/SB/08s filed February 18, 2005, November 21, 2005, and July 1, 2006.

II. Claims 37-40, 43, 44, 48 and 50 Are Definite Under 35 U.S.C. § 112, second paragraph

At page 3 of the Office Action, the Office rejects claims 37-40, 43, 44, 48 and 50 under 35 U.S.C. 112, second paragraph, as allegedly being indefinite.

Solely to advance prosecution, Applicants herewith voluntarily amends claims 37, 38 and 48. The amendments do not change the scope of claims 37, 38, and 48, and claims dependent thereon. Applicants' amendments overcome the rejection of claims 37-40, 43, 44, 48 and 50.

Withdrawal of the rejection under 35 U.S.C. § 112, second paragraph, is therefore kindly requested.

III. Claims 37-40, 43, 44, 48 and 50 Are Properly Described Under 35 U.S.C. § 112, First Paragraph

The Office rejects claims 37-40, 43, 44, 48 and 50 under 35 U.S.C. 112, first paragraph, as allegedly failing to comply with the written description requirement.

Solely to advance prosecution, Applicants herewith voluntarily amends claims 37, 38 and 48. The amendments do not change the scope of claims 37, 38, and 48, and claims dependent thereon. Applicants' amendments overcome the rejection.

Withdrawal of the rejection of claims 37-40, 43, 44, 48 and 50 under 35 U.S.C. § 112, second paragraph, is therefore kindly requested.

IV. Claims 37-40, 44 and 48 Are Novel Under 35 U.S.C. § 102(b)

At page 7 of the Office Action, the Examiner rejects claims 37-40, 44 and 48 under 35 U.S.C. § 102(b) as allegedly being anticipated by GenBank® GI:16565115. The Examiner asserts that claim 37 is anticipated by GenBank® GI:16565115 because the reference discloses a nucleotide sequence which includes a "gene fragment represented by SEQ ID NO.:3."

Solely to advance prosecution, Applicants herewith voluntarily amends claims 37, 38 and 48. The amendments do not change the scope of claims 37, 38, and 48, and claims dependent thereon. Applicants' amendments overcome the rejection.

Withdrawal of the rejection under 35 U.S.C. § 112, second paragraph, is therefore kindly requested.

Regarding the § 102(b) rejection based on cited reference Random Primer 24, Applicants traverse the rejection on the ground that the cited reference does not disclose Applicants' invention (i.e., fragments of SEQ ID NO.:3). Cited Random Primer 24 fails to disclose any

particular sequence or provide functional data that Applicants' fragments are disclosed. The manufacturer cites to Rothstein et al. (attached herewith) as evidence that Random Primer 24 can be used for "universal detection and visualization of DNA fragments." However, at page 4158 of Rothstein et al., Figure D, line 3, only a single, darkly exposed band is shown. One of ordinary skill in the art would not appreciate that Random Primer 24, based on the disclosure of New England Biolabs and Rothstein et al., hybridizes to all possible species of DNA when the data relied on by New England Biolabs discloses only a single intense band of less than 123 bp. Thus, the Examiner's assertions lack support and appear to be hypothetical in nature. The reference is therefore insufficient to qualify as an anticipatory reference under § 102(b).

Furthermore, the Office relies on calculations which use as their basis an average DNA base pair weight which the Examiner halves in order to obtain the molecular weight of an single stranded hypothetical 24-mer. One of ordinary skill in the art recognizes that the weight of DNA bases are known and are used in calculating a polynucleotide weight. One of ordinary skill in the art appreciates that averaging leads to errors in a calculation, such that the result obtained is incorrect. Thus, one of ordinary skill in the art would understand that the Examiner's conclusion that nine copies of each and every possible 24-mer is inconclusive.

Withdrawal of the rejection under § 102(b) is therefore kindly requested.

In view of the above, reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,

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Neurobiology

Chronic inhibition of superoxide dismutase produces apoptotic death of spinal neurons

(amyotrophic lateral sclerosis/antioxidant/free radicals/motor neuron/organotypic culture)

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Communicated by Paul Talalay, January 14, 1994 (received for review November 30, 1993)

ABSTRACT **Mutations in the gene for Cu/Zn superoxide dismutase (SOD1) have been detected in some families with an autosomal dominant form of amyotrophic lateral sclerosis; these mutations appear to reduce the activity of this enzyme. To determine whether decreased SOD activity could contribute to motor neuron loss, SOD1 was inhibited chronically with either antisense oligodeoxynucleotides or diethyldithiocarbamate in spinal cord organotypic cultures. Chronic inhibition of SOD resulted in the apoptotic degeneration of spinal neurons, including motor neurons, over several weeks. Motor neuron loss was markedly potentiated by the inhibition of glutamate transport. In this paradigm, motor neuron toxicity could be entirely prevented by the antioxidant *N*-acetylcysteine and, to a lesser extent, by the non-*N*-methyl-D-aspartate glutamate receptor antagonist 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine hydrochloride. These data support the hypothesis that the loss of motor neurons in familial amyotrophic lateral sclerosis could be due to a reduction in SOD1 activity, possibly potentiated by inefficient glutamate transport.**

Amyotrophic lateral sclerosis (ALS) is a motor neuron disease characterized clinically by progressive weakness, wasting of muscles, and spasticity due to the slow loss of lower motor neurons in the spinal cord and in the neocortex. Familial ALS represents 5–10% of all cases and is virtually indistinguishable clinically from the more common sporadic form. Mutations in the Cu/Zn superoxide dismutase (SOD1) gene on chromosome 21 have been detected in some families with the autosomal dominant form of familial AML (1, 2). Initial studies indicate that these mutations reduce the activity of SOD1 (2, 3). This protein is a homodimeric metalloenzyme that catalyzes the dismutation of the superoxide anion to oxygen and hydrogen peroxide (4). Excessive levels of oxygen radicals, such as superoxide anion, have been implicated in neuronal injury, either directly or through the formation of more reactive oxygen species such as hydroxyl radicals (5–7). However, it has not been established that a chronic reduction in SOD1 activity diminishes the viability of spinal motor neurons. To investigate this, we have developed a model of slow toxicity in cultured organotypic spinal cord slices which combines the advantages of long-term survival with partially preserved synaptic connections (8). In this model, SOD activity was inhibited by the use of either antisense oligodeoxynucleotides (ODNs) or metal-chelating agents such as diethyldithiocarbamate (DDC), which has been shown to potentiate oxygen radical-induced toxicity in acute preparations (5).

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METHODS

Organotypic Spinal Cord Cultures. Eight-day-old neonatal rat pups were decapitated, and the spinal cords were rapidly harvested and cultured (8). Culture medium, including any added pharmacological agent, was changed twice weekly. With this technique, >95% of the explants can be maintained in culture for >3 months with excellent organotypic cellular organization. Test drugs were added 8 days after cultures were prepared.

Motor neuron survival was measured by counting motor neurons in cresyl violet-stained cultures. Motor neurons were operationally defined as any neuron larger than 30 μm in the ventral horn and were counted as described (8).

Biochemical Assays. To determine choline acetyltransferase (ChAT) activity, the spinal cord tissue in each dish (five slices) was pooled and frozen at −75°C until assay. Each culture well represented one time point or drug concentration, and ChAT activity for each time point was calculated from the mean ± SEM of two to eight replicate culture wells. ChAT activity was measured radiometrically with [^{3}H]acetylcoenzyme A (Amersham) (9). Protein content of tissue homogenates was determined by a Coomassie G-250 assay (Pierce).

Total SOD activity was measured in sonicated tissue homogenates (1–200 μg of protein) (10). The amount of SOD activity in experimental samples was compared with that in age-matched control cultures.

Antisense ODN Treatment. Organotypic cultures were prepared from 8-day-old rat lumbar spinal cords (8). After 8 days in culture, phosphorothioate ODN was added to the culture medium. Culture medium was changed twice weekly and included fresh phosphorothioate ODN. Cultures were treated with either sense or antisense ODN; the antisense sequence, 5′-CACACGGCCTTCGTCGCCATAACTCGCTAG-3′, spans from 10 bases 5′ to 17 bases 3′ of the start codon (11, 12). Control cultures received either no ODN or sense ODN. Cultures were then assessed in three ways: (*i*) total SOD activity was measured at selected time points in tissue homogenates, (*ii*) ChAT activity was measured in sense- and antisense-treated cultures, and (*iii*) motor neurons were counted in treated cultures stained with cresyl violet. For biochemical assays, each culture was gently removed from the culture well and frozen at −75°C until assay.

Abbreviations: ALS, amyotrophic lateral sclerosis; SOD1, Cu/Zn superoxide dismutase; DDC, diethyldithiocarbamate; NMDA, *N*-methyl-D-aspartate; ChAT, choline acetyltransferase; U83836E, (−)-2-[[4-(2,6-Di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]methyl]-3,4-dihydro-2,5,7,8-tetramethyl-2*H*-1-benzopyran-6-ol dihydrochloride; GYKI-52466, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine hydrochloride; ODN, oligodeoxynucleotide.

†To whom reprint requests should be addressed.

Apoptosis and DNA Fragmentation. Apoptotic nuclei were identified in cresyl violet-stained whole cultures by the characteristic condensed chromatin and nuclear fragments (13, 14). Apoptotic cells were quantified at weekly intervals by examining 10 random high-power (×250) fields from cultures stained with cresyl violet. Each time point represents the mean ± SEM of apoptotic nucleus counts from two to eight cultures. Apoptotic nuclei were rarely observed in age-matched control cultures. For confirmation, cultures were stained with Hoechst 33258 (Calbiochem) to identify condensed chromatin (15). Cultures were fixed with 4% paraformaldehyde, stained with Hoescht 33258 (10 μg/ml in phosphate-buffered saline) for 10 min, washed, mounted, and observed with a Zeiss Axiovert fluorescence microscope.

To examine DNA fragmentation, DNA from pooled spinal cord cultures was extracted (15) and then electrophoresed in 1.5% agarose in buffer containing ethidium bromide, along with a 123-bp-ladder standard. To improve visualization of the DNA fragments, the DNA was transferred to GeneScreen membrane (DuPont) and hybridized for 18 hr at 42°C with a universal 36-mer ODN primer (New England Biolabs) labeled at the 3' end with fluorescein-conjugated dUTP (Amersham). Blots were washed under low-stringency conditions [twice for 5 min in 5× standard saline citrate (SSC)/0.1% SDS at room temperature and once for 15 min in 1× SCC/0.1% SDS at room temperature] and incubated with anti-fluorescein-conjugated horseradish peroxidase, followed by an enhanced chemiluminescence detection system (Amersham). Blots were exposed to Kodak XAR-5 autoradiographic film for various times.

Drugs. *threo*-Hydroxy-DL-aspartate, DDC, *N*-acetylcysteine, and α-tocopherol were from Sigma. (−)-2-[[4-(2,6-Di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]methyl]-3,4-dihydro-2,5,7,8-tetramethyl-2*H*-1-benzopyran-6-ol dihydrochloride (U83836E) was kindly provided by E. Hall (Upjohn). 1-(4-Aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine hydrochloride (GYKI-52466) was generously supplied by I. Tarsawa (Institute for Drug Research, Budapest).

RESULTS

Organotypic cultures were prepared from rat postnatal lumbar spinal cord and incubated long-term with various doses of DDC (Fig. 1*A*). Both 1 mM and 5 mM DDC potently inhibited SOD activity in these organotypic spinal cord cultures [by 82 ± 4% (mean ± SEM) and 100 ± 1%, respectively], whereas 0.1 mM DDC inhibited SOD activity by <25%. The viability of motor neurons was monitored either by measuring culture ChAT activity, which is largely restricted to motor neurons in the lumbar spinal cord (16, 17), or by counting motor neurons in cultures stained with cresyl violet (8). DDC at 1 mM became progressively neurotoxic after 4 weeks in culture, as measured by loss of ChAT activity and by counts of large ventral-horn neurons (Fig. 1 *A* and *B*). DDC at 5 mM was progressively neurotoxic beginning within 1 week after chronic administration. We were unable to detect any morphological or biochemical evidence that low-dose DDC (0.1 mM) was toxic to motor neurons during the 1 month study period (Fig. 1*A*). Chronic inhibition of SOD with 1 mM DDC was toxic to both motor neurons and other types of neurons in the cultures (Fig. 1*E*).

Aside from inhibiting SOD, DDC has a number of properties which could contribute nonspecifically to its toxicity. For example, DDC has weak reducing properties (9). To control for this effect, organotypic cultures were maintained long-term with low concentrations of two potent reducing agents, dithiothreitol and thioglycolate (18). Neither dithiothreitol (10 μM) nor thioglycolate (250 μM) produced significant motor neuron toxicity when maintained for up to 1 month in culture (Fig. 1*C*).

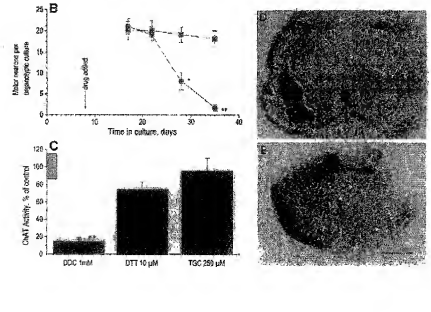

FIG. 1. Neurotoxicity produced by chronic inhibition of SOD1 in organotypic cultures of lumbar spinal cord. (*A*) ChAT activity in spinal cord organotypic cultures as a marker for motor neuron toxicity in the presence of DDC. Cultures were prepared from lumbar spinal cords, and after 8 days in culture, DDC at 0.1 mM (■), 1 mM (●), or 5 mM (▲) was added to the culture medium. These drug concentrations were maintained in all subsequent medium changes. DDC-induced motor neuron toxicity was dose dependent. Shaded bar on the *y* axis represents control mean ± SEM. (*B*) Counts of spinal cord motor neurons in control cultures (■) and cultures treated chronically with 1 mM DDC (●). Motor neuron loss occurred concurrently with loss of ChAT activity. (*C*) Comparison of effects of 4 weeks of DDC (1 mM), dithiothreitol (DTT, 10 μM), or thioglycolate (TGC, 250 μM). For all experiments, statistical significance was tested by Student's independent *t* test comparing drug vs. control: *, $P < 0.05$; **, $P < 0.01$. (*D*) Five-week-old control culture stained with cresyl violet. Arrow indicates ventral motor neuron. (*E*) Loss of ventral motor neurons and generalized cell loss in culture treated for 4 weeks with 1 mM DDC. (Bar = 400 μm.)

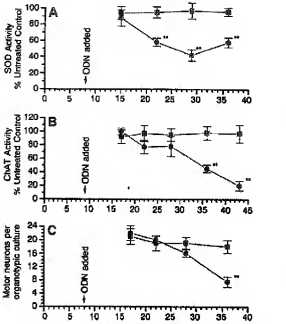

FIG. 2. Effect of long-term treatments with antisense (●) or sense (■) ODN in organotypic cultures prepared from lumbar spinal cord on SOD activity (*A*), motor neuron survival as reflected by tissue ChAT activity (*B*), and cell counts of large ventral horn motor neurons >30 μm in diameter in cresyl violet-stained whole cultures (*C*). Sense ODN had no effect on SOD activity, ChAT activity, or motor neuron survival. Partial inhibition of SOD by antisense ODN produced mild motor neuron toxicity as reflected by a parallel drop in ChAT activity and motor neuron cell number (*B*). Each point represents the mean ± SEM of two to six replicate culture wells, each containing five spinal cord slices. Statistically significant differences of antisense ODN treatment vs. control (independent *t* test): **, $P < 0.01$.

In addition to inhibiting SOD1 activity, DDC can also decrease glutathione levels in some cell cultures (19). We therefore studied the effects of two other copper-chelating agents which potently inhibit SOD while having little effect on glutathione levels (19). Both agents, triethylenetetramine (1 mM) and *tetrakis*(2-pyridylmethyl)ethylenediamine (100 μM), produced >50% loss of ChAT activity after 1 and 3 weeks, in culture, respectively. It therefore seems unlikely that the effects of DDC on motor neurons were a consequence of a decrease in glutathione.

It is also possible that DDC could adversely affect neurons in these cultures by inhibiting cytochrome oxidase (complex IV) in mitochondria (20, 21). Because this possibility was difficult to exclude, we conducted parallel experiments to inhibit SOD1 more specifically, using a phosphorothioate antisense ODN corresponding to the 5′ region of the SOD1 mRNA. The 30-mer SOD1 antisense ODN, extending from 10 bases upstream of the start codon to 17 bases downstream (11), was administered at 5 μM to the organotypic cultures twice a week to inhibit synthesis of SOD1 (12). Control cultures were treated with a matching 30-mer sense ODN at 5 μM. Antisense ODN produced a long-term 40–55% inhibition of SOD1 activity which was maximal by 3 weeks in culture (Fig. 2*A*) (14). Treatment with sense ODN had no effect on SOD1 activity.

To determine whether chronic inhibition of SOD1 by antisense ODN decreased neuronal viability, spinal cord organotypic cultures were incubated with antisense ODN as before. At selected times, ChAT activity was measured to evaluate motor neuron toxicity (Fig. 2*B*), and motor neurons

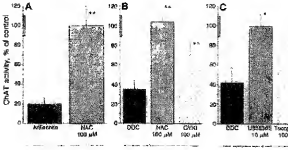

FIG. 3. Neuroprotective properties of the antioxidants U83836E, *N*-acetylcysteine (NAC), and α-tocopherol or the non-NMDA antagonist GYKI-52466 on motor neuron toxicity mediated by SOD inhibition. After 4 weeks in culture with 5 μM antisense ODN (*A*), or after 4 weeks in culture with 1 mM DDC (*B*), or after 1 week in culture with 5 mM DDC (*C*), spinal cord tissue was assayed for ChAT activity. Protection against motor neuron toxicity by the various antioxidants suggests that the neurotoxicity is mediated in large part by oxidative stress. Results are presented as a percentage of ChAT activity in age-matched untreated control cultures. For all experiments, culture medium, along with added pharmacological agents, was changed twice weekly. Control cultures were also treated with the putative protective drugs alone; there were no significant effects of the neuroprotective agents alone, or of sense ODN, on ChAT. Each time point represents the mean ± SEM from three to six replicate wells, each containing five slices. Shaded y-axis bars represent control mean ± SEM. The significance of a drug's protective effect was tested by the independent *t* test comparing drug plus DDC or antisense ODN vs. DDC or antisense ODN alone: *, $P < 0.05$; **, $P < 0.01$.

were counted directly in cresyl violet-stained cultures (Fig. 2*C*). After treatment of cultures with SOD1 antisense for 4 weeks, significant neurotoxicity was evident as assessed by reductions both in ChAT activity and in numbers of motor neurons per culture (Fig. 2 *B* and *C*).

To determine whether the neurotoxicity of SOD inhibition was due to chronic oxidative stress, organotypic spinal cord cultures were cultured with a variety of antioxidants in combination with antisense ODN or DDC. *N*-Acetylcysteine (100 μM), which can act as an antioxidant, either directly or as a precursor for the antioxidant glutathione (22, 23), provided almost complete protection against oxygen radical toxicity produced by antisense ODN and 1 mM DDC (Fig. 3 *A* and *B*). When a higher dose of DDC (5 mM) was used to inhibit SOD (Fig. 3*C*), α-tocopherol (100 μM) and the antioxidant U83836E (10 μM), but not *N*-acetylcysteine (data not shown), partially protected against motor neuron toxicity.

It has been hypothesized that motor neuron death in familial ALS may result from SOD mutations in concert with glutamate-induced excitotoxic injury (24). This suggestion is in accord with autopsy studies documenting diminished glutamate uptake in brain homogenates from patients with sporadic ALS (25) and our report that chronic inhibition of glutamate transport with *threo*-hydroxyaspartate or pyrrolidinedicarboxylic acid is toxic to motor neurons in organotypic cultures (8). To determine whether glutamate could exacerbate the oxidative stress induced by DDC, we maintained our organotypic cultures with a nontoxic concentration of DDC (0.1 mM) in combination with a low concentration of *threo*-hydroxyaspartate (100 μM). This combination of glutamate-transport blockade and DDC markedly potentiated motor neuron death as gauged by accelerated loss of ChAT activity (Fig. 4).

This interaction of glutamate and oxidative stress was explored further with neuroprotective glutamate receptor antagonists. Excess glutamate can lead to the formation of oxygen radicals, through actions at either *N*-methy-D-

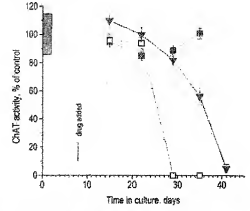

FIG. 4. Potentiation of motor neuron toxicity by inhibition of glutamate uptake. Organotypic cultures prepared from lumbar spinal cord were incubated with either 0.1 mM DDC (■), 100 μM *threo*-hydroxyaspartate (▼), or DDC (0.1 mM) combined with *threo*-hydroxyaspartate (100 μM) (□). Medium, along with added drugs, was changed twice weekly. Motor neuron toxicity occurred earlier and was more severe with glutamate uptake inhibition superimposed on SOD inhibition. Shaded bar on y axis indicates control mean ± SEM.

aspartate (NMDA) or non-NMDA receptors (26, 27). Our previous studies with organotypic cultures demonstrated that glutamate-induced motor neuron toxicity was completely mediated by non-NMDA glutamate receptors (8). For the present experiments, when the noncompetitive, non-NMDA antagonist GYKI-52466 (100 μM) was incubated chronically with cultures exposed to 1 mM DDC, GYKI-52466 provided a small but significant degree of protection against the neurotoxic effects of baseline glutamate in our cultures (Fig. 3*B*). Whether the source of extracellular glutamate in these experiments was neurotransmitter release or leakage from dying cells is not known.

The morphology of neurons chronically exposed to DDC or to SOD1 antisense ODN was quite distinctive, with several features indicative of apoptosis, including dense chromatin masses, condensation of chromatin at the nuclear membrane, and nuclear fragmentation (Fig. 5 *A–C*) (13, 14). In addition, DNA from DDC-treated organotypic cultures was degraded in a pattern resembling the oligonucleosomal "ladder" seen after endonuclease activation (Fig. 5*D*) (15). Temporally, the appearance of neurons with these features paralleled the development of SOD inhibition and the loss of ChAT activity (Fig. 5*E*). Cells with this apoptotic appearance were rarely observed in cultures subjected to chronic inhibition of glutamate uptake by *threo*-hydroxyaspartate (data not shown); in such cultures the major morphological feature was early cellular vacuolization and late necrosis (8, 28). The selectivity of neuronal loss for motor neurons was not formally studied. However, after 3–4 weeks of treatment with antisense ODN, there was a clear loss of motor neurons in cultures which had evidence of only mild overall injury.

DISCUSSION

These studies demonstrate that chronic reduction in SOD1 activity is toxic to motor neurons *in vitro*. A corollary implication is that reduced SOD1 activity in ALS patients with SOD1 mutations may also be directly toxic to motor neurons *in vivo*. Because SOD1 is ubiquitously expressed throughout all tissues, including those in the central nervous system (29), it is likely that some factor other than SOD1 mutations themselves accounts for the selective degeneration of motor neurons in familial ALS. Our data indicate that one

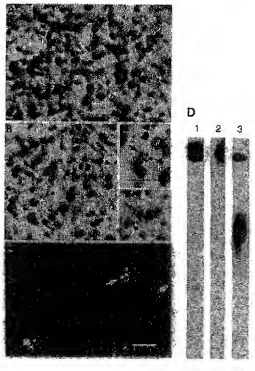

FIG. 5. Chronic inhibition of SOD with DDC or antisense ODN produces apoptotic cell death in organotypic spinal cord cultures. As before, cultures were prepared from 8-day-old rat lumbar spinal cord, and after 8 days in culture, DDC (0.1, 1, or 5 mM) or antisense ODN (5 μM) was added to the culture medium and maintained in all subsequent medium changes. (*A*) Untreated 1-month-old control culture stained with cresyl violet. (*B*) Numerous apoptotic cells in a culture treated for 1 month with 1 mM DDC. (*Insets*) High-power view of typical chromatin clumping after treatment for 1 month with 1 mM DDC (*Upper*) or 1 month with antisense ODN (*Lower*). Arrows indicate apoptotic nuclei. (Cresyl violet staining; *Inset* bar = 25 μm.) (*C*) Fluorescence microscopy of culture treated for 1 week with 5 mM DDC and stained with Hoechst 33258, demonstrating brightly staining apoptotic nuclei (arrow) with condensed chromatin and nuclear fragments. (Bar in *C* for *A–C* = 100 μm.) (*D*) Electrophoretic analysis of DNA from cultures treated with DDC. Lane 1, 123-bp DNA ladder standard; lane 2, control organotypic culture; lane 3, spinal cord cultures treated for 1 month with 1 mM DDC. (*E*) Counts of apoptotic cells from cultures treated for 4 weeks with 0.1 mM DDC (▲), 1 mM DDC (●), or 5 μM antisense ODN (□) compared with untreated control organotypic spinal cord cultures (■). Sense ODN treatment had no effect compared with untreated control cultures. Significance for DDC- or antisense ODN-treated cultures vs. untreated cultures: *, $P < 0.05$; **, $P < 0.01$.

such factor may be the excitotoxic influence of the neurotransmitter glutamate. Inhibition of glutamate transport markedly potentiated the toxicity of low levels of the compound DDC. These observations suggest that impaired glutamate transport, or even baseline glutamatergic activity, may target motor neurons and contribute to their loss in ALS. Other oxidative insults in motor neurons [e.g., hydroxyl radicals and peroxynitrite (30) or other metabolic stresses] might also contribute to motor neuron death.

The motor neuron death induced by the SOD1 antisense ODN was associated with a reduction in SOD1 activity of about 50%. This is approximately the level of SOD1 activity loss detected in tissues of familial ALS patients with SOD1 mutations. Because dominantly inherited diseases are not generally associated with a loss of function (31), it remains possible that the SOD1 mutations in familial ALS patients are lethal because they confer some novel, cytotoxic function on the enzyme in addition to reducing its SOD activity.

Chronic SOD1 inhibition appears to cause apoptotic death of neurons which is reparable by antioxidants in our cultures. One implication of this finding is that excessive free radicals, presumably including the superoxide anion, trigger a sequence of events culminating in endonuclease activation. This is consistent with reports that oxidative stress can induce apoptosis both in embryonic neurons and in neural cell lines (32, 33). Conversely, in some neural lines, overexpression of SOD1 diminishes apoptosis (D. E. Bredesen, personal communication). Our data also suggest the hypothesis that motor neuron death may be apoptotic in familial ALS patients with SOD1 mutations. This is potentially of therapeutic importance, as some neurotrophic factors (NGF, BDNF, NT-3, and CNTF) as well as other proteins (e.g., BCL2) can rescue primary neurons from apoptotic cell death (35, 36).

To what extent the loss of SOD1 in cultures treated with antisense ODN produced selective motor neuron toxicity was not explored in this study, but preliminary observations suggest that motor neurons are more sensitive to oxygen radical toxicity than other neurons in the organotypic spinal cord culture.

The neuroprotective effect of both a non-NMDA glutamate receptor antagonist and antioxidants in this model suggests that these agents may be beneficial in the treatment of ALS. The positive results of recent, preliminary clinical trials with riluzole (37), a drug that alters glutamatergic neurotransmission (34, 38, 39), appear to validate this therapeutic approach.

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